

Article

Use of Gelatin as Tannic Acid Carrier for Its Sustained Local Delivery

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ABSTRACT

Background: Polyphenols are macromolecules that play a pivotal role in plant protection against external aggression from microorganisms or radiations. Their antimicrobial properties have raised interest in the medical community for several years now. Among them, tannic acid is one of the cheapest polyphenols extracted from plants. However, its broad use is impeded because it is unstable, with rapid hydrolysis leading to byproducts such as epigallocatechin with less activity. In this work, we developed a porous material to deliver locally tannic acid.

Methods: The preparation of this matrix relied on the interaction between gelatin and tannic acid in an aqueous medium. Structure properties of the developed matrix were investigated through rheology measurement and by scanning electron microscopy (SEM). Tannic acid quantity and release over time were measured. Antimicrobial activity was tested in vitro on *E. coli* and *S. aureus*.

Results: The presence of two tannic acid populations, one free to diffuse across the scaffold and the second stabilized by gelatin with a more structural activity, was demonstrated. We showed that the formulation is possible above a molar ratio of 1:15 (gelatin:tannic acid). Gelatin was used here as a carrier for the tannic acid. Tannic acid content could be modulated depending on its initial concentration during the preparation. Tannic acid was released from these matrices over a period of four days at a concentration that was above the minimum inhibitory concentration (MIC). Antimicrobial activity was confirmed on *E. coli* and *S. aureus*. The porous material could be conserved by lyophilization without loss of activity. Its handiness is good enough to consider its use as a wound dressing.

Conclusions: We demonstrate herein an easy and cost-effective protocol to locally deliver tannic acid.

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KEYWORDS: gelatin; tannic acid; biocompatible materials; biomedical engineering

ABBREVIATIONS

G, gelatin; TA, tannic acid; SEM, scanning electron microscopy; MH, Mueller-Hinton; MIC, minimum inhibitory concentration; CMC, critical micellar concentration

INTRODUCTION

Polyphenols are macromolecules that play a pivotal role in plant protection against external aggression from microorganisms or radiations [1]. The family is broad with epigallocatechin, gallic acid derivatives, tannins, flavonoids, isoflavonoids, and anthocyanins to cite only a few. Their activity against bacteria, viruses, and fungi have raised interest in their use in medicine. Additional functionalities such as their antioxidative properties and anti-cancer activity, which have been studied previously, help to motivate the research on their use in clinical applications. While polyphenols are available by the simple consumption of fruits, vegetables, and wine or coffee, many efforts have been put into their isolation and presentation to be readily used in the clinic under other forms. Polyphenols of various origins have been incorporated into various materials, usually without real isolation of the active compound. For example, Talón et al. incorporated pea starch and thyme extract into chitosan sponges for their antioxidant activity [2]. The isolation of polyphenols can be time consuming and expensive, making them poor candidates for materials formulation. Among them, tannic acid is of particular interest. It is readily available and cheap. It presents good antimicrobial activity on several bacteria and antioxidant properties. However, it is quite unstable with a rapid degradation into smaller macromolecules such as gallic acid in an oxidative environment. Its oxidation tends to diminish its potential as an antimicrobial agent. It can also be cytotoxic depending on its concentration. Hence, it has to be delivered at a concentration high enough for antimicrobial activity but low enough to avoid a deleterious effect on tissues. Research on the controlled release of tannic acid is of great importance. Up to now, few examples of materials incorporating tannic acid and intended for controlled release have been published. Lu et al. prepared chitosan–gelatin sponges loaded with tannins and platelet-rich plasma as a wound dressing to activate wound healing. Tannic acid plays a role in crosslinking the sponge and to some extent added antimicrobial properties [3]. Guo et al. developed a gelatin/TA sponge crosslinked with AgNO₃ as a bioadhesive with antimicrobial properties [4]. TA is known to interact strongly with gelatin, mostly via the proline residues of the protein [5]. Other strategies aimed at grafting of tannic acid via their hydroxyl groups onto the carboxyl group of polymers via esterification methods to produce

poly(methacrylic acid) microparticles releasing TA for more than 40 h [6]. Tannic acid-based particles were successfully produced from the macro- to nanosize range. Such particles incorporated in porous poly(HEMA) cryogels helped to add antimicrobial properties to this future wound dressing material [7]. Apart from its biological properties, tannins in general and tannic acid in particular are well known for their interactions with proteins. They strongly interact with proteins via hydrogen bonds. This explains why their use in the leather industry was so prominent. It can be used as a crosslinking agent to stabilize nanoparticles or scaffolds. However, its use has to be modulated to insure the functionality of the materials. Indeed, by depositing tannic acid and alkaline phosphatase on substrates layer-by-layer, we previously showed that alkaline phosphatase was inactivated when the concentration of tannic acid was increased [8].

In this study, we developed a method for the local delivery of tannic acid using gelatin (G) as a carrier. It resulted in a material that could be used for example as a wound dressing. Tannic acid was used both for the shaping of the gelatin matrix and as a therapeutic agent. The preparation process relied on the interaction of tannic acid with the protein to form a precipitate when raised above a threshold in the tannic acid:gelatin molar ratio. We showed that these matrices after crosslinking with formaldehyde can be manipulated and are stable up to seven days. More interestingly, the designed matrices showed a slow release of tannic acid. Such wound dressing materials present a good antimicrobial activity. Their conservation was ensured by lyophilization.

MATERIALS AND METHODS

Materials

Tannic acid (CAS number 1401-55-4, ref 403040-500G), gelatin type A with an isoelectric point close to 9 (Gelatin from porcine skin CAS number 90000-70-8, ref 1890-1009), citric acid (CAS number 77-92-9, ref 251275), sodium phosphate dibasic (CAS number 7558-79-4, ref S7907), and Luria Bertani broth (ref L3022) were purchased from Sigma-Aldrich (St. Louis, MI, USA). Mueller–Hinton Broth (BD™DIFCO™, ref BD 275710) and Mueller–Hinton gelose (BD™DIFCO™, ref BD 214530) were purchased from Fisher Scientific (Hampton, NH, USA).

G/TA Matrix Preparation

All preparations were made in a citrate/phosphate buffer at pH = 7.00 to work in close physiological conditions. Citrate/phosphate buffer was prepared by mixing 65.88 mL of Na₂HPO₄ 0.5 M solution, 7.06 mL citric acid 0.5 M solution, and 127.1 mL of MilliQ water. TA and gelatin solutions were prepared just before the TA/gelatin matrix preparation. The TA solutions were prepared at various concentrations depending on the TA:gelatin molar ratio by dissolving TA powder in citrate/phosphate buffer and stirring for 1 h to ensure the complete dissolution of TA. The solubility

of tannic acid in water is $100 \text{ mg}\cdot\text{mL}^{-1}$ at ambient temperature. TA has an isoelectric point close to 8.5 [9] and is hence non-charged in the experimental conditions where the TA/gelatin blends were prepared. The gelatin solutions were prepared at 2 or 10 mg/mL depending on the size of the matrix prepared. Gelatin solutions were heated at 37°C to ensure complete dissolution. Once dissolved, the gelatin solution was cooled to room temperature before use. G/TA matrices were prepared by mixing equal volumes of TA and gelatin solutions followed by 30 s of vortexing. The obtained solution was poured in a mold or in a well of a multiwell plate and centrifuged at 1200 rpm for 15 min in a plate centrifuge (Allegra X-15R centrifuge, rotor SX4750, Beckman Coulter). The supernatant was discarded and replaced by an equal volume of paraformaldehyde (PFA) 4% solution in citrate/phosphate buffer at $\text{pH} = 7.00$. G/TA matrices were incubated for 24 h at room temperature. The PFA solution was then discarded and the matrix rinsed three times with an equal volume of citrate/phosphate buffer at $\text{pH} = 7.00$ for at least 15 min each. The obtained matrices were used directly or lyophilized. Before lyophilization, the matrices were frozen at -80°C for at least 3 h. Lyophilization was then performed with a CHRIST Alpha 1-4 LD plus lyophilizator (Martin Christ Freeze Dryers GMBH, Osterode am Harz, Germany), for 24 h at -56°C and 0.5 mBar. Lyophilized G/TA matrices were kept at room temperature and protected against moisture before use. G/TA matrices were prepared at different molar ratios and are described as G/TA_x , with x corresponding to the ratio of TA compared to gelatin. As gelatin is polydisperse, its average molecular weight of 80 kDa, as indicated by the furnisher, was used for the calculations.

G/TA Matrix Characterization

Scanning electron microscopy

G/TA_x matrices were first lyophilized as described above. They were then sectioned in the middle and fixed on a holder to allow imaging their cross section or their surface. The G/TA_x matrices were then coated with a gold–palladium alloy using a Hummer JR sputtering device (Technics, CA, USA). Scanning electron microscopy was then performed with a Quanta 250 ESEM (FEI Company, Eindhoven, The Netherlands) with an accelerating voltage of the electrons of 5 kV.

TA adsorption isotherm

G/TA_x matrices were prepared at various molar ratios of TA, ranging from 1:15 to 1:100, with a constant concentration of gelatin (2 mg/mL). For each molar ratio, the TA content in the matrix formed was measured. This measurement was performed by means of a solution depletion method. After completion of the centrifugation step (cf. Section “**G/TA Matrix Preparation**”), the TA content (in mg) of the supernatant was measured by UV-Visible spectroscopy at $\lambda = 277 \text{ nm}$ with a SAFAS mc²

spectrophotometer (Xenius, SAFAS, Monaco). At this wavelength, gelatin that is devoid of L-tyrosine, L-phenylalanine, and L-tryptophan residues does not absorb light. This allows quantification of TA without interference from the protein. Measurement was done on 100 μ L solution transferred into a 96-well plate (UV star, ref 655801, Greiner Bio-One). TA concentration in the supernatant was calculated from the OD measurement using a calibration curve performed in the same conditions. The TA content in the matrix (TA_m), in mg, was calculated with Equation (1)

$$TA_m = TA_0 - TA_s, \quad (1)$$

where TA_0 is the quantity of tannic acid used during the matrix preparation and TA_s is the quantity of TA measured in the supernatant after completion of the centrifugation. TA_m was plotted as a function of TA_0 to get an adsorption isotherm.

Rheology

Oscillatory amplitude shear experiments were performed on a Kinexus Rheometer (Malvern Instruments Ltd., MA, USA). Cylinder-shaped gelatin/tannic acid matrices with 34 mm diameter and 4 mm thick were prepared on a circular silicon sheet. Frequency sweep measurements were performed at 10 Hz, which is in the region of linear response determined previously by sweep frequency measurement. Measurement were performed with a cone-plate geometry with a cone size of 4 cm and an angle of 4°.

Measurement of Tannic Acid Release from the G/TA Matrices

Tannic acid release from the matrices was measured by UV-Visible spectroscopy. After preparation of the G/TA_x matrices, they were incubated at room temperature in citrate/phosphate buffer at pH = 7.00. The release of TA was measured in the buffer supernatant at various time points. One hundred microliters were withdrawn, and the absorbance measured at $\lambda = 277$ nm with a SAFAS mc² spectrophotometer. Measurements were performed in a 96-well plate (UV star, ref 655801, Greiner Bio-One). The amount of released tannic acid was calculated from a calibration curve performed in the same conditions. At each time point measured, the buffer was completely withdrawn and replaced by fresh one. The results are expressed as a cumulative release.

Antimicrobial Activity

Bacteria

Staphylococcus aureus (S. aureus, ATCC 25923) and *Escherichia coli* (E. coli, ATCC 25922) microorganisms were cultured according to the manufacturer's instructions in their respective media: Luria Bertani broth was used for E. coli, while Mueller-Hinton broth (MHB) was used for S. aureus.

Antimicrobial activity measurement

Agar diffusion assay

The bacteriostatic effect of tannic acid was evaluated by the agar diffusion assay [10]. The inoculum used for this experiment was prepared using an overnight culture of *S. aureus* or *E. coli* in MH broth. The turbidity was adjusted to a 0.5 McFarland. This well diffusion test was performed using three identical series, distributed in two Petri dishes containing 25 mL of Mueller–Hinton broth and bacteriological agar. Nine wells, 3 mm in diameter, were cut out of the agar. One hundred microliters of bacteria suspension were spread homogeneously over each agar petri dish. Forty microliters of TA solution (10, 5, 2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 mg/mL) were placed into each well. The Petri dishes were incubated at 37 °C for 24 h. Diffusion distances were determined as half the inhibition zone diameter minus the well diameter. Petri dishes were analyzed individually to determine the minimum inhibitory concentration (MIC) and the average values from three repeats were taken for the determination of the final MIC. MIC was calculated using a model based on a generalized diffusion equation that includes a dissipative term [10].

Growth inhibition in planktonic culture

Antimicrobial activity measurements were performed on matrices prepared in 48-well plates from 1 mg of gelatin. Bacterial strains were cultured aerobically at 37 °C in a Mueller–Hinton broth (MHB) at pH 7.4. One colony was transferred to 10 mL of MHB medium and incubated at 37 °C for 20 h. Five hundred microliters of bacterial solution, representing 4.10^5 CFU, were added to each well. Plates were then incubated for 24 h at 37 °C under constant stirring. After completion of the incubation period, 100 μ L of medium was withdrawn and transferred into a 96-well plate. The absorbance at $\lambda = 600$ nm was then recorded using a Multiskan EX microplate spectrophotometer (ThermoFisher Scientific) to quantify bacterial growth. Percentage of growth was expressed regarding the positive control represented by an empty well.

RESULTS

G/TA_x Matrix Characterization

G/TA_x matrices were prepared based on the protocol described in Figure 1. A rapid mixing of TA and gelatin solution leads to gelatin precipitation that can be easily centrifuged to form a matrix.

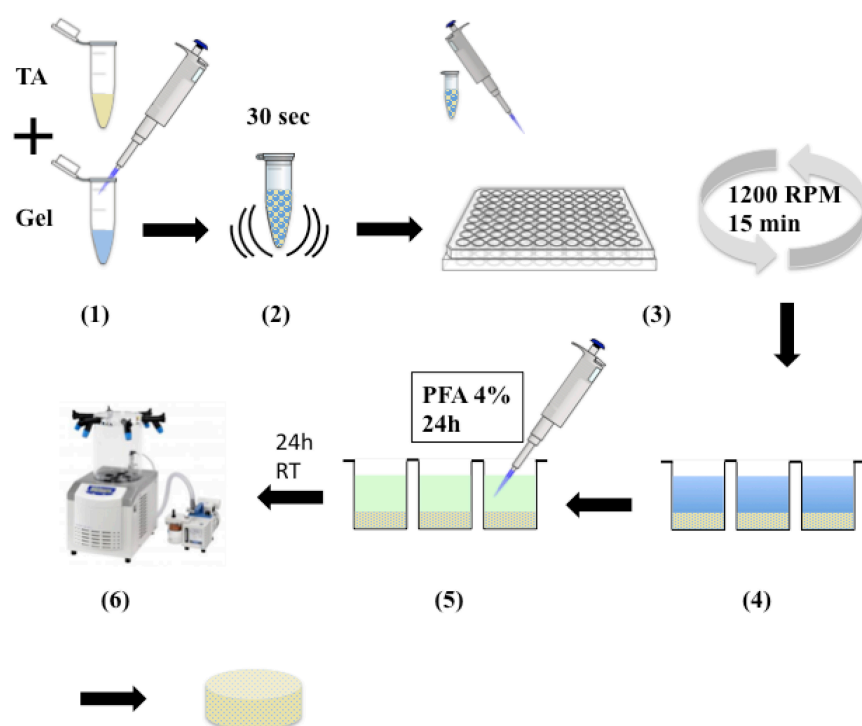


Figure 1. G/TA_x matrices preparation: (1) Gelatin solution in citrate/phosphate buffer at pH = 7.00 and tannic acid solution in citrate/phosphate buffer at pH = 7.00 are mixed at equal volume. (2) G/TA blends are thoroughly mixed for 30 s (3), and then dispensed into a suitable container and centrifuged for 15 min at 1200 rpm (4). (5) G/TA_x matrices are incubated in PFA 4% solution in citrate/phosphate buffer at pH = 7.00 for 24 h at room temperature. After three rinsing steps, the matrices are lyophilized (6).

Both the quantity of gelatin used and the shape of the mold determine the size and shape of the matrix. We crosslinked the matrix after it was centrifuged to stabilize it. Indeed, protein–tannic acid interactions are non-covalent, mainly hydrogen bond and hydrophobic interactions, and can be destabilized by several parameters such as pH or osmotic pressure. The range of molar ratios tested was from 1:15 to 1:200.

Scanning electron microscopy micrographs of the matrix after lyophilization showed that the obtained matrix is porous. The pores seem to be interconnected. However, more interestingly, the walls of the matrix show a granular aspect at high magnification (60,000×) (Figure 2).

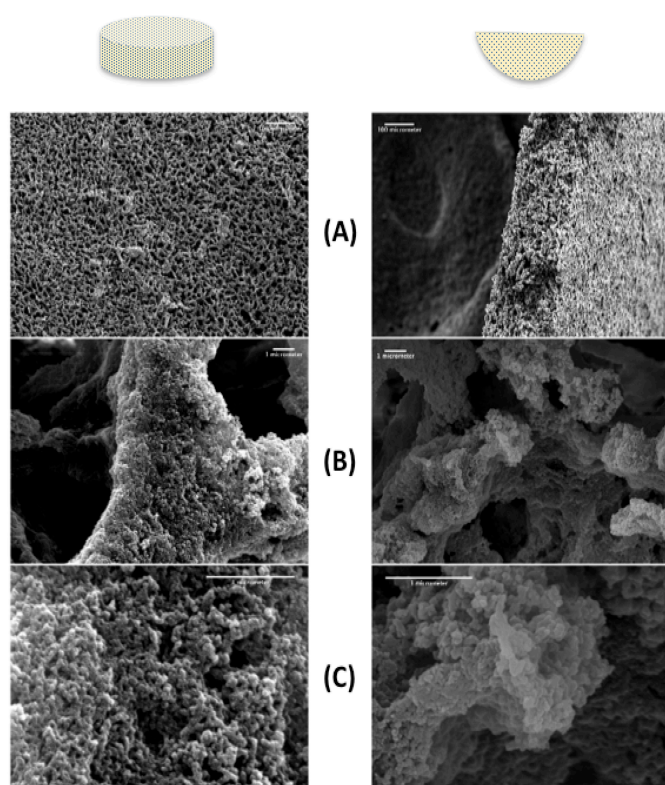


Figure 2. SEM micrograph of a G/TA₇₀ at: 200× (A); 2000× (B); and 60,000× (C). Observations were performed on the surface of the matrix (left) or on the section (right). Scale bar = 100 mm (A) and 1 mm (B,C).

The effect of the crosslinking step on the matrix was assessed by measuring the rheological properties of the matrix as a function of time. As shown in Figure 3, we measured an increase of the storage (G') and loss (G'') moduli with time from the beginning of the crosslinking process. After 2 h, those two parameters remained stable.

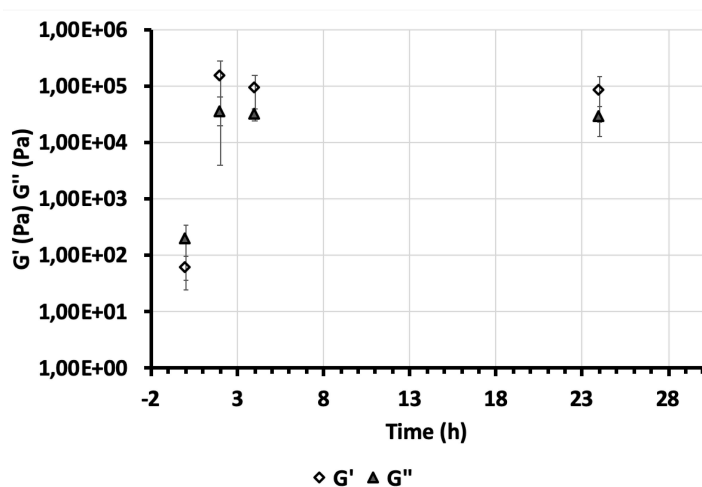


Figure 3. Evolution of G' and G'' during the crosslinking process. The data correspond to the average over three measurements and the error bars correspond to ± 1 standard deviation.

These results were confirmed by the evaluation of the compressive modulus before and after crosslinking. The compressive modulus

increased from 46 ± 4 to 350 ± 50 kPa upon crosslinking. This showed that the crosslinking of the gelatin molecules with PFA was effective and changed the overall physical properties of the matrix. Measurement of the compressive modulus did not show any difference between Day 0 and Day 7 of storage in citrate/phosphate buffer at pH = 7.00 (data not shown). The water content of G/TA₁₀₀ and G/TA₁₅₀ matrices corresponded to $84 \pm 5\%$ of the mass of the matrix, as determined by weighing the hydrated and dried samples.

As we consider gelatin as a TA carrier, we evaluated the quantity of polyphenol held by gelatin as a function of the initial tannic acid concentration. The obtained results are presented in Figure 4. In the range of tested concentrations, we did not observe saturation of the adsorption isotherm with a visible plateau. TA concentration can therefore be tuned by adjusting the TA concentration during the formation of the matrices.

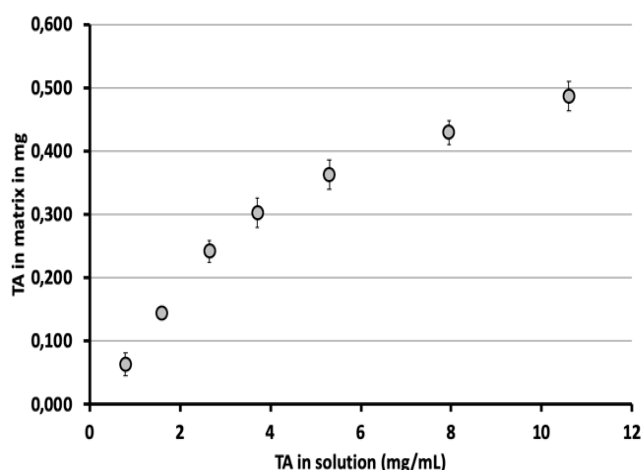


Figure 4. Measure of the tannic acid content in G/TA_x as a function of the initial concentration of tannic acid used during the formulation process for a fixed concentration of gelatin at 1 mg/mL. The data correspond to the average over three independent measurements and the error bars to ± 1 standard deviation.

The release of TA was measured at various time points during four consecutive days. The results are presented as cumulative tannic acid release in Figure 5A as amount per mg of gelatin or in percentage of initial tannic acid content (see Supplementary Figure S1). The point at Day 1 represents the tannic acid release during the crosslinking step. The point at Day 1.5 represents the concentration of tannic acid that is released during the rinsing step that insured PFA elimination. Days 2–4 represent the release in the conditions of use. For each G/TA_x composition, we observed a release over the time course of the experiment. This release depended on the concentration of tannic acid used during the formulation of the matrix, with a more important release for G/TA₂₀₀ than for G/TA₁₅. For all tested TA concentrations, we observed a pronounced release at Day 1 that represents the diffusion of unbound TA out of the matrix. At Days 2–4 we still observed a TA release but at a slower rate: between 40 and 2 μg for a matrix made from 1 mg of gelatin.

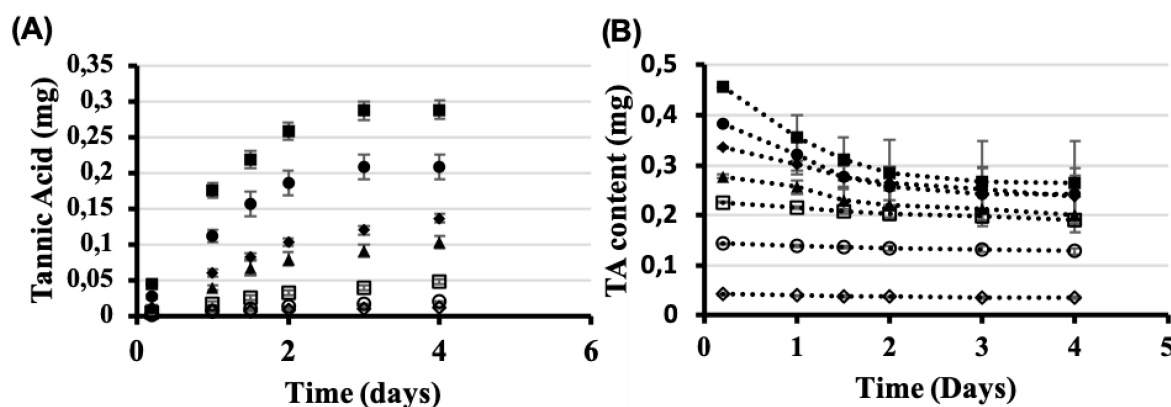


Figure 5. Tannic acid release from G/TA_x matrices over the time: (A) amount of tannic acid delivered from a G/TA_x matrix made from 1 mg gelatin; and (B) amount of tannic acid in the G/TA_x matrix left over the time in mg ± SE. (◇) G/TA₁₅; (○) G/TA₃₀; (□) G/TA₅₀; (▲) G/TA₇₀; (◆) G/TA₁₀₀; (●) G/TA₁₅₀; and (■) G/TA₂₀₀.

The quantities of TA remaining in the G/TA₂₀₀, G/TA₁₅₀, G/TA₁₀₀, G/TA₇₀, and G/TA₅₀ matrices were observed to be nearly the same. This quantity, about 200–250 µg, was greater than the available TA amount in G/TA₃₀ (125 mg) and G/TA₁₅ (33 mg) (Figure 5B). Statistical analysis ($p < 0.05$, ANOVA) showed that the amount of released tannic acid was lower for the matrices prepared with lower molar ratios of polyphenol (G/TA₁₅ and G/TA₃₀). Such a finding suggests that, above a molar ratio of 1:50, the quantity of tannic acid is such that not all the available polyphenol molecules can interact strongly with gelatin and are therefore released.

Antimicrobial Properties of the G/TA_x Matrices

We first determined the antimicrobial properties of tannic acid on the two bacterial strains of interest: *S. aureus* (Gram+) and *E. coli*. (Gram–). Those two bacterial strains are involved in diverse diseases with different clinical expression and are of concern in nosocomial infections. Agar diffusion assays allowed determining the MIC at 105 mg/mL for *E. coli* and 100 mg/mL for *S. aureus*.

Then, we determined the antimicrobial activity of G/TA matrices made with 0.3 mg of gelatine (Figure 6). Over this 24 h experiment, we recorded that the inhibition of bacterial growth depended on the matrices and the bacterial strain tested. For *E. coli*, a complete growth inhibition was observed with G/TA₇₀, G/TA₁₀₀, G/TA₁₅₀, and G/TA₂₀₀. Growth inhibition increased with the increase of the molar ratio (G/TA₁₅ < G/TA₃₀ < G/TA₅₀). The same behavior was recorded for *S. aureus*. For the same molar ratio, we recorded a better growth inhibition of G/TA₁₅, G/TA₃₀, and G/TA₅₀ for *S. aureus* than for *E. coli* with more than 90% inhibition.

Finally, as we are aware that tannic acid, a hydrolysable polyphenol, is not stable in oxidative conditions, we tested the effect of lyophilization on the antimicrobial properties of the G/TA matrices. G/TA₇₀ matrices were prepared as before but followed by a lyophilization process. Before their use, they were kept in dry conditions and protected from light. Their

antimicrobial activity was tested as before. At 24 h, growth inhibition on *E. coli* was the same as previously recorded with fresh G/TA₇₀ matrices (Figure 6 inset).

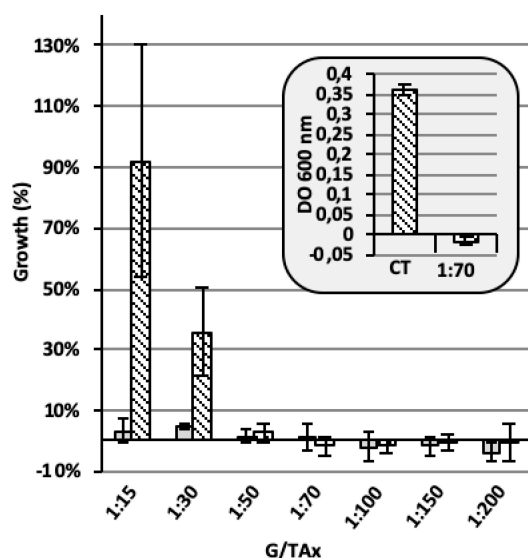


Figure 6. Evaluation of bacterial growth of *E. coli* (dashed bars) and *S. aureus* (plain bars) in presence of G/TA_x matrices. Percentage of growth was determined and compared to normal growth in the same media but without matrix. (Inset) Evaluation of inhibition growth on *E. coli* of G/TA₇₀ matrix after lyophilization.

DISCUSSION

We succeeded in preparing gelatin/tannic acid matrices that represent a cost effective and easy way to produce a tannic acid delivery system. Our strategy was based on the natural interaction of tannic acid with proteins through hydrogen bonds and hydrophobic interactions. With such a method, we were able to produce matrices with gelatin concentration that do not allow gel formation, such as 1% w/v solutions [11]. As the interaction between gelatin and tannic acid relies on non-covalent binding, we can expect, depending on the pH and ionic strength, for example, the release of non-strongly-bound TA [12]. The process seems to be overly simple but different parameters have to be taken into account such as pH and ionic strength because they can affect the interaction between both components and therefore the structuration of the matrix and the release of tannic acid. First, not only hydrogen bonding takes place, but, according to some other studies, hydrophobic and electrostatic interactions also occur. At pH = 7.00, lower than the pK_a value of tannic acid, electrostatic interactions are possible with the negatively charged gelatin of type A (pH_i = 9). However, the ionic strength has to be close to physiological value. Indeed, when ionic strength increases, gelatin takes a more compact conformation due to internal charge screening. It avoids interchain repulsion and favors the interaction with tannic acid [13]. Hydrophobic bonding occurs between L-proline in gelatin and the phenolic group in tannic acid. This interaction mode could generate some cavities in the

materials, similar to what was observed in the construction of gelatin/tannic acid multilayer films [13]. Overall, all these weak interactions have the potential to generate macromolecules mobility. In our case, we observed a modification of the area of our matrices over time (Supplementary Figure S2) with a diminution of 10% after four days in buffer at pH = 7.00 and 35% after 23 days. It shows that the system is not at equilibrium once produced. We observed that the shrinking of the matrix is dependent on the medium, being more pronounced in DMEM, a cell culture media, than in citrate/phosphate buffer. It suggests that the interactions undergone by gelatin are influenced by the nature and concentration of the present electrolytes, as observed in the literature [14].

It has been demonstrated that the interaction of tannic acid with protein is also dependent on its concentration. Under the critical micellar concentration (CMC), the interaction is specific, and we can find few tannic acid molecules interacting with proteins. Above the CMC, tannic acid form aggregates and interaction with proteins are less specific. The interaction of tannic acid aggregates can be done with different protein in their surrounding media. Large TA-protein aggregates can be formed [15]. In our case, the concentration of tannic acid was always above the CMC of tannic acid (3.10×10^{-4} mol/L, i.e., 0.53 mg/mL) [15]. We thus produced large aggregates that could be centrifuged to prepare the material in the form of a pellet. This also explains the porous aspect of the matrix, as found at high magnification in SEM.

We observed a release of tannic acid over time from each formulation. Such a release was observed during about four days with an initial burst release lasting 24 h followed by a slower release up to four days. Considering the quantity of tannic acid left in the matrices after four days, we observed that all matrices made from a molar ratio above 50 between TA and gelatin led to the same amount remaining in the matrix. Hence, above a molar ratio of 50, there are two tannic acid populations: one can be considered as strongly bound to gelatin and the other considered as mobile. Below this threshold value, TA release was minimal. The tannic acid released over time was therefore present in the water content of the matrices or resulted from tannic acid aggregates that disassembled. In fact, TA spontaneously forms solution aggregates at high concentrations [16]. From a practical point of view, using gelatin as a binder for tannic acid implies using molar ratios above 50. Otherwise, we cannot have two populations of tannic acid; only the “structural” one is available with no diffusible molecules. The increase of the G:TA molar ratio allows an increase in polyphenol release.

To help the handling of the matrix by hand or with tweezers (Supplementary Video S1), a crosslinking step was added in the formulation by the use of paraformaldehyde. This crosslinking step increased the mechanical properties, as demonstrated by compressive modulus testing. Paraformaldehyde is a well-known protein crosslinker [17]. However, at first glance, it could appear impractical to use it for

biomaterials, as the toxicity of paraformaldehyde is potent. Formaldehyde and glutaraldehyde are already used in pig cardiac valve preparation. In that preparation, the action on the protein helps to reduce the immune response to the grafts [18,19]. No toxicity has been recorded thus far with the use of these bioprosthesis. Use of other crosslinking agents that are more biocompatible could be of interest, such as genepin [20]. The formaldehyde crosslinking is not stable. Temperature will affect the half-life of the reaction. At 37 °C, the half-life of formaldehyde protein–DNA crosslinks was measured to be about 22 h [21]. Thus, after several days, we can expect that some of the gelatin–gelatin crosslinks no longer persist.

TA is also reported as a crosslinking agent [5,22,23]. It could help the overall structuration of the scaffold. Researchers using tannic as a crosslinker tend to use far lower TA:protein ratios than what we did in this study. At pH = 8.00, covalent binding between tannic acid and gelatin can occur between the amine group of gelatin (lysine, arginine, and histidine residues) and the oxidized phenolic group of tannic acid [24]. However, at this pH, oxidation can occur rapidly and would diminish the antimicrobial activity, which is why we decided to work at a controlled pH = 7.00. Our matrices remained stable for at least 23 days.

Use of tannic acid as an antimicrobial agent tends to be tricky because of its instability. Indeed, under oxidative environment, it degrades into smaller molecular weight products by hydrolysis [25]. Such byproducts lose their antimicrobial properties. In our matrices, oxidation of the tannic acid could be easily seen by a color change from a yellowish color to dark green. The same observation can be made when oxidation is triggered by sodium periodate [8]. For this reason, we tested the antimicrobial activity after lyophilization of the matrix. Indeed, once lyophilized, tannic acid can be conserved for a longer period of time. No loss in antimicrobial activity was recorded with such preparation.

Tannic acid could have some cytotoxicity related to its abilities to interact with membrane, inhibit enzyme activity, and chelate metal especially iron. We tested cytotoxicity on NIH3T3 mouse fibroblasts. The threshold concentration above which we recorded cytotoxicity is 125 μ M (representing 200 mg/mL) (Supplementary Figure S3). Considering the concentration of tannic acid released over time, we are in a range that is not toxic for eukaryotic cells. However, further in vitro testing is mandatory before testing such material in vivo in clinical condition such as wound closure model.

The easy handling of this porous material to give it various shapes and sizes is promising for future use. Indeed, applications could be found in the preparation of wound dressings with anti-microbial properties. The material can be manipulated with tweezers and can then be put in contact with a wound and comply with the actual protocol of wound dressing. Research tends to avoid the use of antibiotics to diminish the appearance of bacterial resistance. For example, we can cite the use of silver derivatives in various materials such as cellulose or carrageenan-based

hydrogels [26,27]. Such cost-effective antimicrobial agents delivering matrices could also be used in periodontology. Indeed, adjuvant therapy to scaling and root planning therapy using a degradable material loaded with chlorhexidine inserted in periodontal pockets is already available in the clinic (Periochip®) [28,29]. The material designed herein could be an alternative in the case of chlorhexidine allergy.

CONCLUSIONS

Herein, we show an easy and cost-effective way to prepare a porous material that can be used to locally deliver tannic acid. Conserved as lyophilized, tannic acid keeps its activity and has been proved to be antimicrobial against *S. aureus* and *E. coli* once released into the surrounding media after matrix hydration. The content of tannic acid can be modulated easily, and thus also the concentration of tannic acid release. It is thus suitable to fight various bacteria even if their sensibility to tannic acid varies. The method allows producing matrices with various shapes and sizes. It therefore could be used in different applications from wound dressing to periodontology.

SUPPLEMENTARY MATERIALS

The supplementary materials on Multilayer Perceptron Network are available online at <https://doi.org/10.20900/pf20200002>.

DATA AVAILABILITY

The dataset of the study is available from the authors upon reasonable request.

AUTHOR CONTRIBUTIONS

FM and VB designed the study. FR and JH performed the antimicrobial experiments. EB, JH and FM performed the matrix characterization. FM and VB analyzed the data. FM and VB wrote the paper with input from all authors.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCES

1. Sileika TS, Barrett DG, Zhang R, Lau KHA, Messersmith PB. Colorless Multifunctional Coatings Inspired by Polyphenols Found in Tea, Chocolate, and Wine. *Angew Chem Int Ed Engl*. 2013;52(41):10766-70.

2. Talón E, Trifkovic KT, Nedovic VA, Bugarski BM, Vargas M, Chiralt A, et al. Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts. *Carbohydr Polym.* 2017;157:1153-61.
3. Lu B, Wang T, Li Z, Dai F, Lv L, Tang F, et al. Healing of skin wounds with a chitosan-gelatin sponge loaded with tannins and platelet-rich plasma. *Int J Biol Macromol.* 2016;82:884-91.
4. Guo J, Sun W, Kim JP, Lu X, Li Q, Lin M, et al. Development of tannin-inspired antimicrobial bioadhesives. *Acta Biomater.* 2018;72:35-44.
5. Zhang XQ, Do MD, Casey P, Sulistio A, Qiao GG, Lundin L, et al. Chemical modification of gelatin by a natural phenolic cross-linker, tannic acid. *J Agric Food Chem.* 2010;58(11):6808-15.
6. Sahiner N, Sengel SB. Tannic acid decorated poly(methacrylic acid) micro and nanoparticles with controllable tannic acid release and antioxidant properties. *Colloid Surface Physicochem Eng Aspect.* 2016;508:30-8.
7. Sahiner N, Sagbas S, Sahiner M, Silan C. P(TA) macro-, micro-, nanoparticle-embedded super porous p(HEMA) cryogels as wound dressing material. *Mater Sci Eng C Mater Biol Appl.* 2017;70(Pt 1):317-26.
8. Allais M, Meyer F, Ball V. Multilayered films made from tannic acid and alkaline phosphatase with enzymatic activity and electrochemical behavior. *J Colloid Interf Sci.* 2018;512:722-9.
9. Oćwieja M, Adamczyk Z, Morga M. Adsorption of tannic acid on polyelectrolyte monolayers determined in situ by streaming potential measurements. *J Colloid Interf Sci.* 2015;438:249-58.
10. Bonev B, Hooper J, Parisot J. Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. *J Antimicrob Chemother.* 2008 Jun;61(6):1295-301.
11. Bello J, Bello HR, Vinograd JR. The mechanism of gelation of gelatin the influence of pH, concentration, time and dilute electrolyte on the gelation of gelatin and modified gelatins. *Biochimica et Biophysica Acta.* 1962 Jan 1;57(1):214-21.
12. Kaspchak E, Mafra LI, Mafra MR. Effect of heating and ionic strength on the interaction of bovine serum albumin and the antinutrients tannic and phytic acids, and its influence on in vitro protein digestibility. *Food Chem.* 2018 Jun 30;252:1-8.
13. Bahmanzadeh S, Ruzgas T, Sotres J. Proteolytic degradation of gelatin-tannic acid multilayers. *J Colloid Interf Sci.* 2018 Sep 15;526:244-52.
14. Shinde UA, Nagarsenker MS. Characterization of Gelatin-Sodium Alginate Complex Coacervation System. *Indian J Pharm Sci.* 2009;71(3):313-7.
15. Cala O, Dufourc EJ, Fouquet E, Manigand C, Laguerre M, Pianet I. The Colloidal State of Tannins Impacts the Nature of Their Interaction with Proteins: The Case of Salivary Proline-Rich Protein/Procyanidins Binding. *Langmuir.* 2012 Dec 18;28(50):17410-8.
16. Allais M, Mailley D, Hébraud P, Ihiawakrim D, Ball V, Meyer F, et al. Polymer-free electrospinning of tannic acid and cross-linking in water for hybrid smart supramolecular nanofibres. *Nanoscale.* 2018;10(19):9164-73.

17. Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K. Chemical and physical basics of routine formaldehyde fixation. *J Oral Maxillofac Pathol*. 2012;16:400-5.
18. Manji RA, Menkis AH, Ekser B, Cooper DKC. Porcine bioprosthetic heart valves: The next generation. *Am Heart J*. 2012 Aug;164(2):177-85.
19. McClurg WM, Lawford PV, Hughes H, Rogers S. Formaldehyde replaces glutaraldehyde in porcine bioprosthetic heart valves. *J Heart Valve Dis*. 1996 May;5(3):343-7.
20. Chen BZ, Ashfaq M, Zhu DD, Zhang XP, Guo XD. Controlled Delivery of Insulin Using Rapidly Separating Microneedles Fabricated from Genipin-Crosslinked Gelatin. *Macromol Rapid Commun*. 2018 May 2;39(20):e1800075.
21. Kennedy-Darling J, Smith LM. Measuring the formaldehyde Protein-DNA cross-link reversal rate. *Anal Chem*. 2014 Jun 17;86(12):5678-81.
22. Fan H, Wang J, Zhang Q, Jin Z. Tannic Acid-Based Multifunctional Hydrogels with Facile Adjustable Adhesion and Cohesion Contributed by Polyphenol Supramolecular Chemistry. *ACS Omega*. 2017 Oct 31;2(10):6668-76.
23. Rubentheren V, Ward TA, Chee CY, Tang CK. Processing and analysis of chitosan nanocomposites reinforced with chitin whiskers and tannic acid as a crosslinker. *Carbohydr Polym*. 2015 Jan 22;115:379-87.
24. Zhang X, Do MD, Casey P, Sulistio A, Qiao GG, Lundin L, et al. Chemical Modification of Gelatin by a Natural Phenolic Cross-linker, Tannic Acid. *J Agric Food Chem*. 2010 Jun 9;58(11):6809-15.
25. Buzzini P, Arapitsas P, Goretti M, Branda E, Turchetti B, Pinelli P, et al. Antimicrobial and antiviral activity of hydrolysable tannins. *Mini Rev Med Chem*. 2008 Oct;8(12):1179-87.
26. Percival SL. Antimicrobial Efficacy of a Silver Impregnated Hydrophilic PU Foam. *Surg Technol Int*. 2018;32:67-74.
27. Zepon KM, Marques MS, da Silva Paula MM, Morisso FDP, Kanis LA. Facile, green and scalable method to produce carrageenan-based hydrogel containing in situ synthesized AgNPs for application as wound dressing. *Int J Biol Macromol*. 2018 Jul 1;113:51-8.
28. Puri K, Dodwad V, Bhat K, Puri N. Effect of controlled-release Periochip™ on clinical and microbiological parameters in patients of chronic periodontitis. *J Indian Soc Periodontol*. 2013;17(5):605-11.
29. Stabholz A, Shapira L, Mahler D, Gellman Y, Ramon T, Dolev E, et al. Using the PerioChip in treating adult periodontitis: an interim report. *Compend Contin Educ Dent*. 2000 Apr;21(4):325-8, 330, 332 passim, quiz 338.

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